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**Note**

# Purification of stroma-free haemoglobin by ATP—agarose affinity chromatography*

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Affinity chromatography has been shown to be one of the most powerful purification techniques available for the purification of biomacromolecules. Mammalian haemoglobin is known to have a diphosphoglycerate (DPG) binding site [1] and avian haemoglobin is known to have an inositol pentaphosphate binding site [2]. This binding site has been found by X-ray crystallography to be localized within a central cavity of the haemoglobin tetramer [3, 4]. The binding of the polyphosphate allosterically affects the conformation of haemoglobin, leading to a lowered oxygen affinity [5]. Conversely, binding of oxygen or carbon monoxide to the haeme moiety results in an allosteric conformation change which in turn lowers the affinity of the haemoglobin for the polyanions [6]. Oxyhaemoglobin is known to bind DPG and ATP weakly [7]. This communication explores the utility of this binding specificity for polyanionic ligands in the isolation and purification of haemoglobin.

Results are presented to show that oxyhaemoglobin forms a reversible complex with agarose– adenosine 5′-triphosphate (ATP—agarose). This binding has shown to lower the oxygen affinity of haemoglobin. The haemoglobin—ATP—agarose complex dissociates in the presence of ATP and other anions. The application of polyanion affinity chromatography to the isolation and purification of haemoglobin, from sources other than outdated blood such as "used" blood from surgery, human placentae and those produced by genetic engineering, for scale-up production of haemoglobin-based blood substitute is discussed.

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## EXPERIMENTAL

ATP—agarose gel, Types 2, 3 and 4, were purchased from Pharmacia P-L Biochemicals (Dorval, Quebec, Canada).

The stroma-free haemoglobin (SFH) was prepared from outdated red blood cells that were washed three times with 150 m*M* sodium chloride (1:2, v/v) followed by lysis with 4 vols. of 5 m*M* phosphate buffer (pH 7.4). To remove stroma and membrane fragments, the haemolysate was filtered by a Pellicon cassette system (Millipore, Boston, MA, U.S.A.) with a fluorocarbon polymer filter (HVLP, porosity 0.5 $\mu$m) followed by a polysulphone filter (100 000 molecular weight cut-off). The haemoglobin in the filtrate was then concentrated to 10 g/dl with a 30 000 molecular weight cut-off membrane Pellicon cassette system. The resulting haemoglobin solution was dialysed against three changes of 50 m*M* Bis—Tris buffer, pH 7.0 (buffer A), sterilized by filtration through a 0.22-$\mu$m Millipore filter and stored at 4°C until use [8].

For the oxygen-binding studies, after washing the ATP—agarose column with 10 bed vols. of buffer A, a small amount of the SFH bound to the gel was resuspended in 2 $\mu$l of buffer A. Its oxygen dissociation curve was obtained with a Hem-O-Scan (SLM-Aminco, Urbana, IL, U.S.A.) oxygen dissociation analyser, and its $P_{50}$ (the partial pressure of oxygen at which half the haemoglobin is in the oxy state) was read directly from this curve. As a control, SFH mixed with plain agarose gel was suspended in buffer A and its oxygen dissociation curve and $P_{50}$ were obtained in the same way.

The elution profiles of SFH were obtained by applying 10 $\mu$l (100 $\mu$g) of SFH to an analytical glass capillary column packed with 10 $\mu$l of ATP—agarose Type 4 gel. The capillary column (50-$\mu$l disposable micropipettes; Drummond, Broomall, PA, U.S.A.) was adjusted to ca. 5 mm in length to give full-scale peak intensity by the UV monitor equipped with a 405-nm filter. The chromatogram was obtained with a fast protein liquid chromatographic (FPLC) system (Pharmacia, Dorval, Canada).

To obtain the cellulose acetate electrophoretic analysis of the haemoglobin solutions, 250 $\mu$l (20 mg) of the SFH solution were then applied to a Pharmacia HR 5/5 column (50 mm × 5 mm I.D.) packed with 1.0 ml of different types of ATP- agarose gels equilibrated with buffer A. The column was first washed with 10 bed vols. of buffer A at a flow-rate of 0.5 ml/min until the eluent was clear, yielding the unretained fraction. ATP (10 m*M* ATP in buffer A) was then introduced into the red gel via a linear gradient to elute the retained fraction. Both the unretained and retained fractions were collected, concentrated and dialysed against buffer A. Cellulose acetate electrophoresis was conducted for each of the fractions in barbital buffer, pH 8.8 (high-resolution buffer, Gelman Sciences, Ann Arbor, MI, U.S.A.).

## RESULTS AND DISCUSSION

### *Haemoglobin—ATP—agarose complex*

Three types of ATP—agarose preparations with different linkages between the ligand and the matrix (Types 2, 3 and 4) were equilibrated with excess

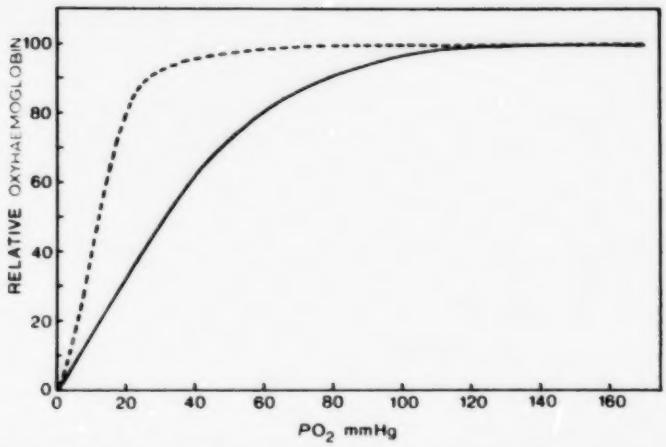


**Fig. 1. Modification of the oxygen affinity of SFH by ATP–agarose gel. Oxygen dissociation curve of SFH in the presence of ATP–agarose gel (solid line) and plain agarose gel (broken line) in 50 m*M* Bis–Tris buffer (pH 7.0) at 37°C.**

oxyhaemoglobin and washed with 10 bed vols. of buffer A until the eluent was optically clear. The oxygen dissociation curves of the red gels were measured with the Hem-O-Scan. The three types of ATP–agarose gels used in the present study are with ATP attached to agarose through the N-6 amino group (Type 2), the 8-position of adenosine (Type 3) or the periodate oxidized ribose moiety (Type 4). Fig. 1 shows a typical oxygen dissociation curve of haemoglobin bound to ATP–agarose Type 4, with that of haemoglobin in the presence of agarose shown as a control. The $P_{50}$ of the control haemoglobin in the presence of agarose is 12 mmHg, while the haemoglobin–ATP–agarose complex has a $P_{50}$ of 35 mmHg. The oxygen dissociation curves of haemoglobin in the presence of the other two types of ATP–agarose gels (Types 2 and 3) give $P_{50}$ values of 35 mmHg each.

These results suggest that oxyhaemoglobin has sufficient affinity for ATP–agarose to form a stable complex with it. Binding of the ATP moiety to the DPG binding site lowers the oxygen affinity of haemoglobin to a level comparable with that of haemoglobin in the presence of four molar equivalents of ATP ($P_{50}$ = 32 mmHg). Thus the spacer group between ATP and agarose is sufficiently long in all three types of the ATP–agarose gels to permit favourable complex formation between the haemoglobin DPG binding site with the ATP moiety, with similar allosteric effects on oxygen binding affinity.

*Binding specificity of haemoglobin to ATP–agarose*

Fig. 2 shows a typical elution profile of haemoglobin loaded onto an analytical capillary column packed with ATP–agarose Type 4 and eluted with or without 10 m*M* ATP in buffer A. In the absence of ATP, the majority of SFH binds to the ATP–agarose gel and causes it to turn red, only a minor fraction is not bound to the gel which appears as the unretained fraction (peak a). The introduction of ATP as a linear gradient results in the elution of haemoglobin bound to ATP–agarose gels as the retained fraction (peak b).

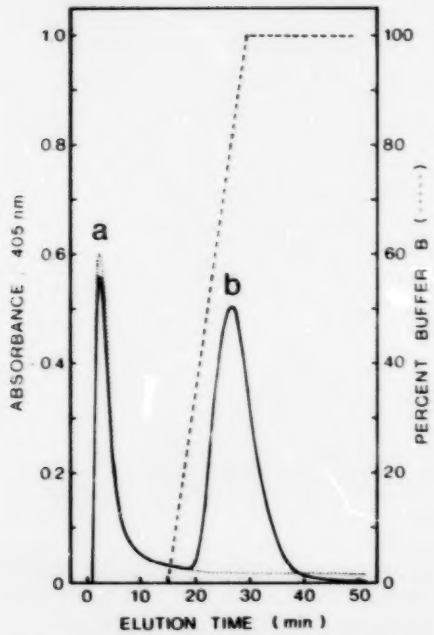


Fig. 2. Elution profile of haemoglobin chromatographed on an ATP—agarose affinity column. The dotted line indicates the elution of SFH by buffer A (50 m*M* Bis- Tris, pH 7.0) only, giving the unretained fraction (peak a). The solid line indicates the elution of SFH by buffer followed by a linear gradient of buffer B (10 m*M* ATP in buffer A: gradient shown as a broken line), giving unretained and retained fractions (peaks a and b, respectively). Experimental conditions were: flow-rate 0.1 ml/min, temperature ~ 20°C.

The specific displacement of SFH by ATP-containing buffer indicates that oxyhaemoglobin is specifically retained via binding of the ATP moiety of the gel to its polyanion binding site. Similar results were obtained using Types 2 and 3 ATP—agarose gels. As further evidence of this specificity, haemoglobin is displaced from the ATP—agarose column by the following anions, in order of decreasing effectiveness or increasing elution time: inositol hexaphosphate > ATP ≃ DPG ≃ pyridoxal phosphate > ADP > phosphate ion > chloride ion (results not shown). The concentration of the competing ligands used is 10 m*M* except for phosphate ion (50 m*M*) and chloride ion (150 m*M*). Elution of haemoglobin from the ATP—agarose column using buffers containing any of the above competing anionic ligands yields a purified and concentrated haemoglobin solution.

*Cellulose acetate electrophoretic pattern of ATP—agarose purified haemoglobin*

Stroma-free haemoglobin is known to contain haemoglobin A as well as minor haemoglobin variants and other red blood cell cytoplasmic proteins. Therefore, we have compared the cellulose acetate electrophoretic patterns of SFH to the ATP—agarose gel purified haemoglobin (Fig. 3). Arrows a and b indicate haemoglobin A and other minor components of haemoglobin, and arrows c and d indicate unidentified minor components of SFH that are enriched in the un-

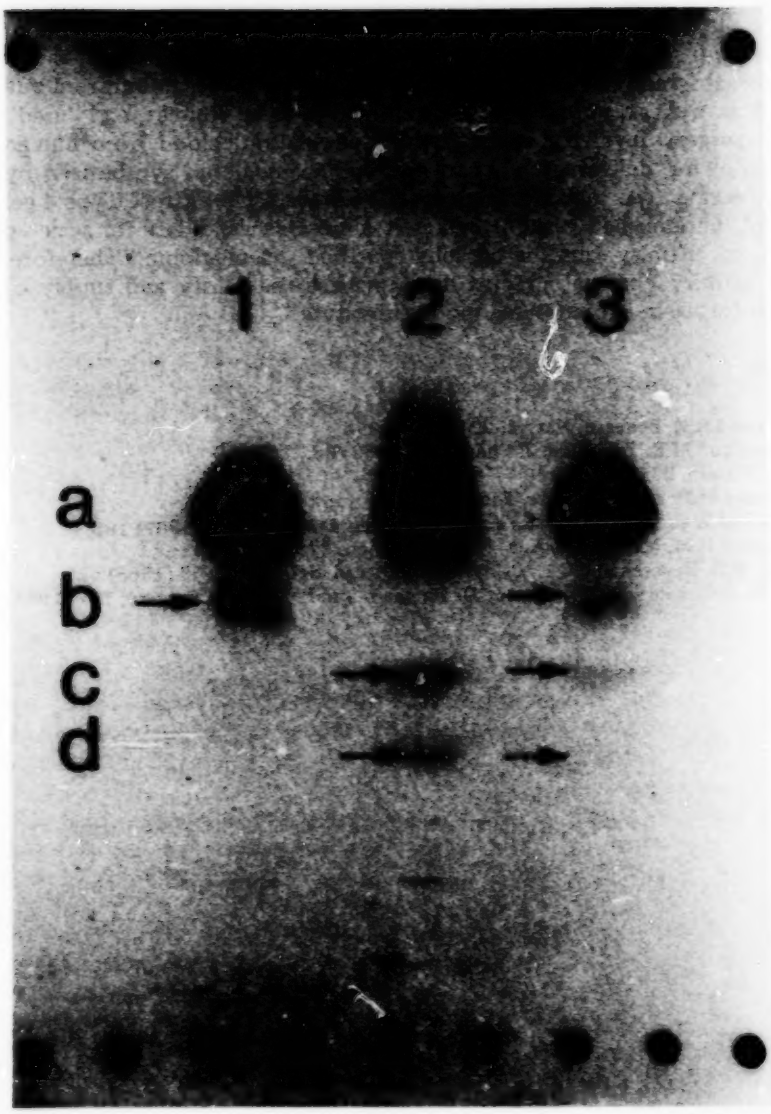


**Fig. 3. Electrophoretic pattern on cellulose acetate of SFH before and after ATP—agarose gel chromatography. Electrophoresis was performed in barbital buffer (pH 8.8). Lanes 1 and 2 are the retained and unretained fractions, respectively (see Fig. 2). Lane 3 is a control sample of the starting SFH. Arrows a and b indicate haemoglobin A and other minor components of haemoglobin; arrows c and d indicate unidentified minor components of SFH that are enriched in the unretained fraction.**

retained fraction. The absence of bands c and d in the retained fraction indicates that ATP—agarose affinity chromatography is capable of further purifying SFH by removing minor components.

A specific purification step is desirable in SFH purification to remove minor components and contaminants. ATP–agarose, in principle, serves as an affinity chromatography system for the purification of haemoglobin from outdated blood as well as, potentially, from unconventional sources, e.g. "used" blood from surgery involving extracorporeal circulation, blood from human placentae following delivery, and, conceivably, haemoglobin synthesized by genetic engineering. The high binding capacity of the ATP–agarose Type 4 gel (≃ 30 mg/ml, unpublished results) makes it a suitable candidate for scale-up preparation of affinity chromatography purified haemoglobin. Therefore, the use of affinity chromatography may increase the purity and supply of haemoglobin for haemoglobin-based blood substitute.

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